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(54) Title: HUMAN NUCLEIC ACID FRAGMENTS, ISOLATED FROM BRAIN ADRENAL TISSUE, PLACENTA OR BONE NARROW

(57) Abstract

This invention provides a nucleic acid fragment encoding a gene product or portion thereof and comprising any one of: (a) a sequence selected from SEQ ID Nos 1 to 1193 from the attached sequence listings; (b) an allelic variation of a sequence as defined in (a); or (c) a sequence complementary to (a) or (b). The invention includes uses of such fragments, and gene products corresponding thereto.

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HUMAN NUCLEIC ACID FRAGMENTS, ISOLATED FROM BRAIN ADRENAL TISSUE, PLACENTA OR BONE NARROW

This invention relates to new nucleic acid fragments encoding gene products or portions thereof, which fragments are obtainable from human nucleic acid populations, individual members of such populations being present in widely varying amounts.

Situations are increasingly arising in which it is necessary to study complex nucleic acid or polynucleotide populations. For example, it is now widely appreciated that an invaluable resource could be created if the entire sequence of the genomes of organisms such as man were determined and the information available. The magnitude of such a task should not, however, be underestimated. Thus, the human genome may contain as many as 100,000 genes [a very substantial proportion of which may be expressed in the human brain (Sutcliffe, Ann. Rev. Neurosci. 11:157 (1988))]. Only a very small percentage of the stock of human genes has presently been explored, and this largely in a piecemeal and usually specifically targeted fashion.

There has been much public debate about the best means of approaching human genome sequencing. Brenner has argued (CIBA Foundation Symposium 149:6 (1990)) that efforts should be concentrated on cDNAs produced from reverse transcribed mRNAs rather than on genomic DNA. This is primarily because most useful genetic information resides in the fraction of the genome which corresponds to mRNA, and this fraction is a very small part of the total (5% or less). Moreover, techniques for generating cDNAs are also well known. On the other hand, even supposing near perfect recovery of cDNAs corresponding to all expressed mRNAs, some potentially useful information will be lost by the cDNA approach, including sequences responsible for control and regulation of genes. Nonetheless, the cDNA approach at least substantially reduces the inherent inefficiencies resulting from analysis of repeated sequences or non-coding sequences in an approach which depends upon genomic DNA sequencing.

Recently, the results of a rapid method for identifying and characterising new cDNAs has been reported (Adams, M.D. et al., Science 252, 1991, pp 1651-1656). Essentially, a semi-automated sequence reader was used to produce a single read of sequence from one end of each of a number of cDNAs picked at random. It was shown, by comparing the nucleic acid sequences of the cDNAs (or the protein sequences produced by translating the nucleic acid sequence of the cDNAs) to each

other and to known sequences in public databases, that each of the cDNAs picked at random, could be unambiguously classified. The cDNAs could be classified as being either entirely new or as corresponding, to a greater or lesser extent, to a previously known sequence. cDNAs identified in this way were further characterised and found to be useful in a variety of standard applications, including physical mapping. Unfortunately, such a process is insufficient. The longer the process is pursued with any given population of cDNAs the less efficient it becomes and the lower the rate of identification of new clones. In essence, as the number of cDNAs which have already been picked rises, the probability of picking a particular cDNA more than once increases. This difficulty is exacerbated by the wide range of abundancies at which different cDNAs can occur, which abundancies can vary by several orders of magnitude. Thus, whereas some sequences are exceedingly rare, a single cDNA type may comprise as much as 10% of the population of cDNAs produced from a particular tissue (Lewin, B. Gene Expression, Vol. 2: Eukaryotic Chromosomes, 2nd ed., pp. 708-719. New York: Wiley, 1980). The need to avoid missing rarer species in any given population presents a considerable problem.

Various approaches (so-called "normalisation" techniques) have been tried in addressing the problem of increasing the efficiency of examination of a mixed nucleotide population, for example, such a population as is to be examined in human genome sequencing.

Thus, a standard PCR protocol can be used to amplify selectively cDNAs which are present at extremely low levels, if there is information about the sequence of those cDNAs. If not, a primer specific to the desired cDNA cannot be constructed and the desired cDNA cannot be selectively amplified. The standard PCR method is therefore inadequate if it is desired to characterise a number of unknown genes.

A second approach involves hybridization of cDNA to genomic DNA. At saturation, the cDNAs recovered from genomic/cDNA hybrids will be present in the same abundance as the genes encoding them. This will provide a much more homogenous population than the original cDNA library, but does not entirely solve the problem. In order to reach saturation in respect of the very rare sequences, it will be necessary to use huge quantities of cDNA, which need to be allowed to anneal to large amounts of genomic DNA over a considerable periods of time. Furthermore, cDNAs which are homologous to genes which are present in multiple copies in the genome will be over-represented.

A third approach exploits the second order reassociation kinetics of cDNA annealing to itself. After a long period of annealing, the cDNAs which remain single stranded will have nearly the same abundance, and can be recovered by standard PCR (see Patanjali, S.R. et al., PNAS USA 88, 1991, pp. 1943-1947; Ko, M.S.H., NAR 19, no.18, 1991, pp 5705-5711). The methods disclosed in these two publications, however, suffer from notable disadvantages. They are entirely dependent on the stringent physical separation of single stranded and double stranded DNA, require an elevated number of manual manipulations in each reaction, and necessitate protracted hybridisation times (up to 288 hours in the method of Patanjali et al.)

Yet a further approach in "normalising" a nucleotide population is described in co-pending British Patent Application No 91 15407.0, filed 17th July, 1991 by MRC, and involves a PCR process in which a mixture comprising a heterogenous DNA population and appropriate oligonucleotide primers is first formed and the DNA denatured, but before effecting a conventional PCR protocol the conditions are altered to allow the denatured strands of the more common DNA species to reanneal together, whilst avoiding annealing of primers to the DNA strands. By this means, rarer species can subsequently be amplified in preference to the more common species.

This PCR normalisation method in general comprises the steps of:

(a) preparing a mixture comprising a heterogenous DNA population and oligonucleotide primers suitable for use in a PCR process, in which the DNA is denatured;

(b) altering the conditions to allow the denatured strands of the more common DNA species to reanneal, while preventing the annealing to the primers to the DNA strands;

(c) further altering the conditions of the mixture in order to allow the primers to anneal to the remaining single-stranded DNA comprising the rarer DNA species; and

(d) carrying out an extension synthesis in the mixture produced in step (c).

Advantageously, the method consists of a cyclic application of the above four steps.

It will be appreciated that the conditions may be altered by the alteration of the temperature of the reaction mixture. However, any conditions which affect the hybridisation of complementary DNA strands to one another may be varied to achieve the required result.

Because the reannealing efficiency of any given DNA species will depend on the product of its concentration and time, the more abundant the sequence the greater the extent to which it will reanneal in any given time period. Once a DNA species has reached a certain threshold concentration it will no longer be amplified exponentially, as a significant amount will have annealed to the double stranded form before the priming step. Thus, as each individual DNA species is amplified by the process to its threshold concentration, the rate of amplification of that species will start to tail off. Eventually, therefore, all DNA species will be present at the same concentration.

The length of the reannealing step will determine how much DNA is present at the threshold concentration. Preferably, therefore, the duration of the reannealing step will be determined empirically for each DNA population.

In the PCR normalisation process in general, the DNA primers may be adapted to prime selectively a sample of the total DNA population. By using primers which will only prime a sample of the population, only that sample will be amplified and normalised. The total quantity of DNA generated will thereby be reduced, which means that the cycling times can be kept low. This ensures that the method is applicable to complex DNA populations such as cDNA populations. In addition, a first primer can be used which is adapted selectively to prime a sample of the total cDNA population, and a second primer which is a general primer. Advantageously, the general primer is oligo dT (each primed cDNA will then be replicated in its entirely, as the oligo dT primer will anneal to the poly-A tail at the end of the cDNA).

In co-pending British Patent Application No 92 14873.3, filed by MRC 13th July, 1992, a new process is described which allows the study and identification of the individual members of a mixed or heterogenous population of nucleotide sequences perhaps of varying abundance. In preferred embodiments of the said process, the starting nucleic acid population is treated by:

(a) subjecting the nucleic acid to the action of a reagent,

preferably an endonuclease which has its cleavage and recognition sites separated, which reagent cleaves the nucleic acid so as to produce double stranded cleavage products the individual strands of which overlap at cleaved ends to leave a single strand extending to a known extent;

(b) ligating the cleavage products from (a) with a population of adaptor molecules to generate adaptored cleavage products, each of which adaptor molecules has a cleavage product end recognition sequence and the population thereof encompassing a range of adaptor molecules having recognition sequences complementary to a predetermined subset of the sequences of the cleavage-generated extending single strands; and

(c) selecting and separating only those adaptored cleavage products resulting from (b) which carry an adaptor of predetermined recognition sequence.

A preferred endonuclease for use in step (a) of the above process is Fok 1.

An important feature of this process is the use of adaptor molecules. The adaptors used must have "overhanging" fragment recognition sequences which reflect or are complementary to the extending cleavage-derived sequences which the adaptors are designed to react with. It is also preferred that the adaptors used should end with a 5' hydroxyl group. The avoidance of a 5' phosphate group removes the risk of inappropriate ligation involving the adaptors.

Adaptor molecules may also contain a portion permitting specific sequence selection and separation (as in step (c) of the process) when a sequence is attached to the adaptor. For example, an adaptor can carry biotin, thereby permitting advantage to be taken of the biotin/avidin
reaction in selecting and separating desired adaptored molecules. Additionally, adaptors preferably comprise a known and selected sequence such that specifically isolated adaptored molecules can be amplified by known techniques (such as PCR) using a primer complementary to the core sequence.

Preferably the adaptors are short double-stranded oligonucleotides which can be joined to the ends of cleavage products. They will have been chemically synthesised so that their sequence can be predetermined

and so that large concentrations can be easily produced. They may also be chemically modified in a way which allows them to be easily purified during the process. As mentioned above, ideally their 5' ends will be unphosphorylated so that once joined to fragments the adaptored end of the latter will no longer be able to participate in further ligation reactions.

It is preferred that the adaptor cleavage product end recognition sequences are on the 5' end of the longest oligonucleotide strand making up the preferred adaptor molecules, are at least 3 nucleotides in length and with totally random bases at the single-stranded position(s) two nucleotides in from the 5' end. This then allows selection to be performed both during the joining reaction and during subsequent priming reactions. Then, because the final degree of selection is a result of the product of the degrees of selection achieved at these two stages, maximum selection can be achieved per adaptor/primer available.

Adaptor strand extensions on the 5' end of the longest oligonucleotide also facilitate the use of modified oligonucleotides for separation purposes. Preferably, the short oligonucleotide will be modified at its 5' end. This has the double benefit of requiring just one modified oligonucleotide for all possible single-stranded extensions that are used, and also placing the modification at a position where it cannot interfere with ligation or subsequent priming reactions.

Although only one type of adaptor is required per ligation reaction, it is preferred that adaptors covering all possible reactions in a chosen subset of sequences be present, because then the opportunity for fragments in the chosen subset to ligate to each other is minimised. It is also preferred that the chosen specific adaptor, carrying a predetermined recognition sequence, should not only be different from the other adaptors in its single-stranded extension, but also different in the rest of its sequence since this allows orientation to be introduced which is useful in subsequent steps. It is therefore also preferred that this adaptor has a modified oligonucleotide to facilitate its separation with the cleaving products to which it joins.

The above "adaptoring" process can be used to generate categories or subsets of sequences by making some of the adaptors specific in some way, and selecting and separating as in step (c). In this way subsets of sequences can be provided depending upon the specific adaptor

chosen, e.g. for use in subsequent nucleotide sequencing. This facilitates, for example, the identification of a large population of sequences by permitting a rational approach to splitting such populations into subsets, each of which subsets can be examined in turn.

In the light of these developments, the present invention now provides a nucleic acid fragment encoding a gene product or portion thereof and comprising any one of:-

(a) a sequence selected from SEQ ID Nos 1 to 1193;

(b) an allelic variation of a sequence as defined in (a); or

(c) a sequence complementary to (a) or (b).

In another aspect, the invention provides a nucleic acid sequence as set out in any one of SEQ ID Nos 1 to 1193, or a complement or allelic variation thereof. Preferred sequences exhibit no more than 90% homology to a human sequence known per se.

In a further aspect, the invention provides a nucleic acid fragment comprising a portion of a sequence as defined above of sufficient size such that a probe of the same size and exhibiting complementarity to said portion can hybridise to said sequence. Preferably, such portions are at least 15 bases in length. It will be appreciated that minor mismatches in the aforesaid "complementarity" are not excluded provided hybridisation can still occur. In general, hybridisation conditions are within the choice of the skilled person, but reference can be made, for example, to the following: Melting temperature of hybrids - Bolton, E. T. and McCarthy, B. J. Proc. Natl. Acad. Sci, 48 p1390 (1962). Effect of formamide on lowering melting temperature - Casey, J. and Davidson, N., Nucleic Acids Res. 4, p1539 (1977). Effect of imperfect homology - Bonner, T. I. et al., J. Mol. Biol. 81, p123 (1973). General - Meinkoth, J. and Wahl, G. Anal. Biochem. 138, p267 (1984). Oligo hybridization and washing - Lathe, R. J. Mol. Biol. 183, P1 (1985).

The present invention also envisages DNA constructs comprising fragments or sequences as referred to above with a control or regulatory sequence.

The invention includes such DNA constructs using a gene system known in the art ligated to a sequence or fragment of the invention so as to enable, upon expression, the provision of a fusion polypeptide. Preferably, an endopeptidase recognition site is provided such that when the sequence or fragment is expressed it is expressed in frame with a known protein with the boundary being a cleavage site for an endopeptidase with a rare cutting site. The known protein can then be affinity purified, and the peptide corresponding to the fragment or sequence in accordance with the invention may be released by the endopeptidase. Alternatively, the whole protein can be used to raise antibodies which can then be screened for those directed at polypeptide corresponding to the fragment or sequence of the invention.

Since the present fragments and sequences can be used to produce, inter alia, corresponding genes, whether by isolating them, by synthesis or otherwise, such use and the resulting DNA fragments comprising genes are further aspects of the invention.

Yet another aspect of the invention is an expression vector comprising a fragment, sequence, gene-comprising DNA fragment, or DNA construct, as above, positioned such that that nucleic acid sequence which encodes the polypeptide corresponding to said fragment, sequence or DNA fragment is in operable reading frame with a control or regulatory sequence.

Other aspects of the invention are host cells incorporating a sequence, or fragment, or gene-comprising DNA fragment, or DNA construct, as above, as a heterologous part of the expressible genetic information of the cell. The production of such modified host cells can be achieved using methods known in the art. Such modified host cells can be used to express corresponding proteins, and these materials lend themselves in turn to the preparation of corresponding monoclonal or polyclonal antibodies using standard techniques.

Also included in this invention are such antibodies. Reference can be made, inter alia, to the following literature: Monoclonal antibodies, Cambell, A. M. Laboratory Techniques in Biochemistry, and Molecular Biology Ed. Burdon, R. H. and van Knippenberg, P. H. vol 13. Elsevier Amsterdam 1984. Goding, J. W., Monoclonal antibodies: Principles and Practice, 2nd Edition, academic Press, London 1986. Kipps, T. J. and Herzenberg, L. A., Handbook of Experimental Immunology : Applications of immunological methods in biomedical sciences, 4th edition Ed. Weir,

D. M. et al., p108 Blackwell scientific Publications, Oxford. Harlow, E and Lane, D. Antibodies, A Laboratory Manual, Cold spring harbor Laboratory, Cold Spring, New York.

Expression in an appropriate higher eucaryotic host may be important to ensure correct protein folding and also activity. Expression to avoid copurification of toxic products can sometimes be better performed in organisms approved for human consumption, eg prokaryotic Bacillus subtilis, eurkaryotic yeast, mammalian cows milk vectors, and other methods known in the art.

The invention also includes novel gene products or portions thereof encoded by a fragment, sequence or gene-comprising DNA fragment of the invention.

It will be appreciated that the sequences of the present invention collectively have utility based, inter alia, upon their common origin, and hence they can effectively be considered together rather than as separate entities. It is convenient to represent them as separate sequences, because this is how they were produced and serves as "punctuation" between the different functional entities which each sequence represents. However, the sequences could just as easily have been presented as a continuous sequence derived by placing them end to end in the order in which they were produced, with a separate indication of where the beginnings and ends of the component sequences are.

In contrast to investigations hitherto, where gene fragments (sequence fragments) could only be identified through some known characteristic [for example: their homology to a fragment which largely encodes amino acids identified by sequencing a previously isolated peptide or is the antisense of that coding sequence; or them having at least partial homology to previously characterised nucleic acids; or them having ability to encode expressed proteins which could later be detected by functional assays of the cells expressing those proteins or by using antibodies which had been previously raised against the proteins to detect their expression, Sambrook J., et al., Molecular Cloning CSH Press 1989], the sequences and fragments described by the present invention are entirely underivable and unpredictable from the prior art, but are nonetheless clearly of great value for various purposes.

Thus, such sequences, by comparing them to sequence databases, can be

used as a means for determining the existence of new members of existing gene families, new human genes when previously only non-human genes were known and new genes when previously no genes were known (Karlin, S. and Altschul, S. F. Proc. Natl. Acad. Sci. 87 p2264-2268 (1980)). In all cases, this allows the isolation of the corresponding genes and their products, and hence enables the manufacture of molecules of potential biological interest by recombinant means. Screening libraries of known materials or hitherto unexplored source materials for biological efficacy is now an important industrial activity in the search for new therapies and therapeutics. When new sequences have already been found to have counterparts in gene families or in non-human genes then knowledge about biological efficacy may already be apparant. For example, new receptors or receptor agonists/antagonists may exhibit differences to known instances of these molecules, and such differences could make them more suitable as therapeutics by, for example, exhibiting binding characteristics which are more in keeping with avoidance of toxicity. Reference can be made, for example, to polymorphic dopamine receptors and the implications for mental health (Iversen, L. Nature 358, p109 (1992), and Van Tol, H. M. M. et al., Nature 358, p149-152 (1992)). Where absolutely required, realisation of full length cDNAs for expression can be achieved by using the sequences to screen (by hybridisation) suitable cDNA libraries containing full length clones (D'Alession, J. M., et al., Focus (Gibco B.R.L) 9 pl (1987)). Alternatively, the sequences can be used to design primers suitable for obtaining the missing sequences by PCR or other amplification methods (Frohman, M. A., Dush, M. K. and Martin, G. R., Proc. Natl. Acad. Sci. 85 p8998-9002 (1988)).

Appropriate use of the sequence fragments in antisense or triple helix (Griffin et al., Science 245 p967-971 (1989)) applications will be useful for identifying manipulable targets related to disease. For example, viruses have been inhibited by antisense RNA to their mRNAs (Chang, L-J., and Stoltfuz, C. M. J. Virol. p921-974 (1987)). A similar effect could be achieved by targetting the expression of cellular proteins which are essential for growth or maintenance of the virus.

Partial or full length cDNAs have great utility once expressed. The manner of expression can be selected by one skilled in the art to suit the intended application. Expression of full length cDNAs is typically required for biological activity. Procaryotic, and lower or higher eucaryotic hosts may be selected as the host for expression and higher

eucaryotes may be preferred to ensure correct modifications, for example, glycosylation in vivo, when this proves to be important. Expression can be ensured by situating the cDNA appropriately to signals for expression (Amann, E. and Brosius, J. Gene 40 p183 1985), Shimuzu, Y et al., Gene 65, p141 (1988), Straus, D. and Gilbert, W. Proc. Natl. Acad. Sci. 82, p2014 (1985)). Such signals may include a promoter for transcription, which may itself be regulatable.

The proteins thus-expressed can be screened for activities of therapeutic or commercial value. It may be that the proteins have to be first isolated for this purpose or can be assayed in situ. It may be desirable that some means of stabilising the expressed protein is employed. This can be achieved, for example, (and as indicated earlier) by expressing in frame as part of a fusion polypeptide (Smith, D. B., et al., Proc. Natl. Acad. Sci. 83 p8073 (1986)).

Useful antibodies can be raised against the expressed proteins. It is commonly not an absolute requirement that full length proteins are produced, although this may influence the quality of the antibodies produced. Peptides as short as 8 or 9 amino-acids in length can be used as antigens (Germain R., N. Nature 353 pp605-607 (1991), Rudensky, A., Y., et al., Nature 353 p622-627 (1991)). Immunogenic peptides could simply be synthesised using the amino-acid sequence translated from a sequence or fragment of this invention. It is desirable, although not absolutely required, that some means of producing purified antibodies is adopted. When fusion polypeptides are used to raise antibodies, an affinity matrix specific for the generic part of the protein allows the fusion polypeptide to be immobilised (Smith, D. B., et al., Proc. Natl. Acad. Sci. 83 p8073 (1986)). The immobilised polypeptide can then be used to affinity purify the antibodies. Antibodies to both the generic part of the fusion polypeptide and the part of interest are produced. When these need to be discriminated between, a different affinity column can be used to remove only those antibodies specific for the generic part of the polypeptide. Alternatively, and as mentioned earlier, it can be arranged that the boundary between the two separate protein components of the fusion polypeptide has the recognition sequence for an endopeptidase with a rare cutting site. The peptide of interest can then be released from the affinity purified polypeptide by the action of the endopeptidase (Nagai, K., and Thogersen, H., C. Methods Enzmol. 153 p461-481 (1987). Another alternative is raise monoclonal antibodies against the purified protein.

The antibodies can be used for localising in situ, or quantifying in samples through, for example, ELISA or RIA assays, peptides against which they were raised. These uses are particularly beneficial when the results of the assays can be correlated to a disease condition, eg cancer. For example tumour markers may be found and used to target therapeutic agents. The antibodies can also be used to detect or monitor markers of undifferentiated growth, infection, cardiovascular or immune disease or a therapeutic response. When the antibodies recognise cell surface proteins they can be used in isolation or in combination to isolate particular populations of cells. These in turn can be used to isolate yet more cDNAs which will be enriched for yet more of such surface markers for the population, which, if similarly screened, will permit yet further subdivision of the population. Ultimately, panels of antibodies which can describe particular disease states will accrue. Such antibodies could be tailored for forensic applications as well as diagnostic purposes and disease monitoring.

The sequences or fragments can also be used for genetic analysis and mapping, for example, to diagnose the likelihood that a given individual is predisposed towards a given genetic disease. In the event of a sequence co-locating, genetically, with a disease gene, it can be used for the derivation of new disease therapies bases upon precise genetic knowledge. Such therapies can include, for example, the techniques of so-called "gene therapy" (Dusty Miller, A. Nature 357 p455-460 (1992)).

Antibodies can be produced against the protein of a genetic disease with sufficient discriminating power to discriminate between diseased and non-diseased states (Caskey, T. Genome Sequencing Conference, Hilton Head, S. Carolina (1991)). This would be useful for reducing the dependence of such tests on nucleic acid-based screens. Such antibodies also have the advantage of allowing detection of faulty expression of the protein, for example levels of expression which may be important for development of the disease in slow onset conditions.

Also very important is that not all cDNAs are likely to be found by conventional means, whereas the present sequences are, in one sense, "comprehensive". The use of the class of cDNAs which corresponds of necessity to truncated clones increases the chances that part of a cDNA will be cloned free of any sequences that could otherwise compromise it from being cloned. Sequence obtained can then be used to generate PCR primers from which the remainder can be obtained without having to

clone.

This invention will now be further described and illustrated by means of the following Examples.

All oligonucleotides used in these Examples were synthesised Trityl on using an ABI 380B DNA Synthesizer according to the manufacturers instructions. Purification was by reverse phase HPLC (see, for example, Becker, C., R., et al., J. Chromatography 326, p293-299 (1985)).

Example 1

Human brain and adrenal tissues were obtained from a mixture of 12 to 15 week menstrual age foetuses and then snap frozen in liquid nitrogen before storing in bijou bottles in a -80°C freezer. The two types of tissue were used separately, directly from the freezer, to prepare cDNA from which restriction fragments were generated for sorting into subsets. 1g portions of each of the separate tissues were homogenised, using an Ultra-Turrax T25 Disperser (Janke and Kunkel, IKA-Labortechnik), on ice in the presence of 4M guanidinium isothiocyanate to solubilise macromolecules. RNA was isolated from each homogenate by using centrifugation to sediment it through caesium trifluoroacetate. This was performed using the Pharmacia kit according to the manufacturer's instructions, except that centrifugation was performed for 36 hours and the RNA obtained was finally desalted and concentrated by performing two ethanol precipitations in succession with two 70% ethanol washes after each precipitation. In each case, polyA$^+$ (mRNA) was isolated from 200 to 400 μg of the total RNA by binding it to magnetic oligo-dT coated beads (Dynal). Solution containing unbound material was removed from the beads, which were washed, and then mRNA eluted directly for use. mRNA isolation was performed in accordance with the manufacturer's instructions. Yields of RNA from the beads were between 1 and 3% of the total RNA. 2 to 4 μg of the eluted RNA were used for cDNA synthesis. cDNA synthesis was performed according to the method of Gubler, U and Hoffman, (B. J. Gene 25 p263 (1983) using a Pharmacia kit according to the manufacturer's instructions. OligodT was used to prime the first strand cDNA synthesis reaction. The cDNA was purified by extracting twice with phenol/chloroform and then low molecular weight solutes including nucleic acids below ca. 300 bases were removed by passing the cDNA reaction mixture through a Pharmacia S400 spun column used according to the manufacturer's

instructions. Running buffer for the column comprised 10 mM TrisHCl, 1 mM EDTA, 50 mM NaCl @ pH 7.5.

The column eluate was adjusted to 10 mM Mg^{2+} and then the purified cDNA was restricted by the action of 1 unit per 10 μl of the endonuclease Fok I at 37°C for 1 hour, so that it would be able to accept adaptors to enable fragment sorting.

The cDNA fragments were purified by two successive phenol/chloroform extractions followed by passing them through S400 spun columns as described above.

The adaptors used were oligonucleotides 5' $N_4N_4N_4N_4$TCCTTCTCCTGCGACAGACA (SEQ ID: 1194) with the complementary strand 5' TGTCTGTCGCAGGAGAAGGA (SEQ ID: 1195) and 5' AAN$_4$N$_4$TCTCGGACAGTGCTCCGAGAAC (SEQ ID: 1196) or 5' TTN$_4$N$_4$TCTCGGACAGTGCTCCGAGAAC (SEQ ID: 1197) each with the complementary 5' biotinylated strand GTTCTCGGAGCACTGTCCGAGA (SEQ ID: 1198). These were added to 25% of the eluted material by incubating together 200 pmoles of the mixture of double-stranded adaptors in the elution buffer to which had been added $MgCl_2$ to 10mM, ATP to 10mM and 0.025 units/μl of T4 DNA ligase. The oligonucleotide 5' biotinylated GTTCTCGGAGCACTGTCCGAGA, (SEQ ID: 1198) and whichever of the complementary oligonucleotides with which it was used, each comprised 1/32 of the molar proportion total adaptors. The final reaction volume was 90 μl which was heated to 65°C for 3 minutes and then cooled to room temperature before the ligase was added. Ligation was performed for 16 hours at 12°C.

Two successive phenol/chloroform extractions were performed to remove the ligase. The final aqueous phase was passed through an S400 spun column (Pharmacia) as described above except that the column was used with 10 mM Tris pH 8.3/50 mM NaCl.

The column eluate was adjusted to 25mM Mg2+, 0.5mM dNTPs in a final volume of 200 μl. The mixture was placed in a thermocycler (Techne MW2) and heated to 78°C for 5 minutes. At this point 10 units of cloned Taq DNA polymerase (AmpliTaq, Perkin Elmer) were added. This was followed by an incubation at 72°C for 10 minutes to fill in the unligated strand of the adaptor. After the second incubation 200 μl of streptavidin coated magnetic beads (Dynal) prepared according to the manufacturers instructions were added to bind cDNA ligated to that of the oligonucleotides which was complementary to the 5'

GTTCTCGGAGCACTGTCCGAGA (SEQ ID: 1198) biotinylated adaptor. Bead binding was allowed to proceed at 28°C for 30 minutes with mixing every 10 minutes.

Un-biotinylated cDNAs were washed from the beads with 400µl each of 2M NaCl twice, fresh 0.15 mM NaOH four times at 28°C for 5 minutes each, water twice and finally a buffer comprising 20 mM Tris pH 8.3, 50 mM NaCl, and 25mM Mg^{2+}. The beads were then resuspended in 240 µl of the final buffer including additionally 0.5 mM dNTPs and divided into 4x60 µl.

Four of the 60 µl aliquots, two from each tissue, were processed further specifically to prime and copy a subset of the immobilised, adaptored fragments. 2 pmoles of the primer 5' CTGTCTGTCGCAGGAGAAGGAA (SEQ ID: 1201) were added to each of two aliquots, one from each tissue. 2 pmoles of the primer 5' CTGTCTGTCGCAGGAGAAGGAG (SEQ ID: 1202) were added to each of the other two aliquots. 2.5 units of Taq DNA polymerase were added to each reaction and 16 cycles of alternate denaturation at 95°C for 30 seconds, annealing at 63°C for 2 minutes and polymerisation at 72°C for 3 minutes was performed to accumulate the selected single-strands in solution.

On completion of the DNA synthesis reactions a further 30 µl of resuspended beads were added to each reaction to remove the biotinylated fragments. The reaction was incubated at 28°C for 30 minutes mixing every 10 minutes to ensure that the biotinylated strands were bead bound. Each aqueous phase containing the newly synthesised strands was then removed and extracted with phenol/chloroform twice to remove the enzyme before being further purified by passing through an S400 spun column equilibrated with 10 mM Tris pH 8.3/50 mM NaCl as described above.

Rounds of PCR amplification of subsets of the selected fragments were performed by using the original primer in each case, together with one of the primers 5' GTTCTCGGAGCACTGTCCGAGAG (SEQ ID: 1199) or 5' GTTCTCGGAGCACTGTCCGAGAC SEQ ID: 1200). This simultaneously rendered the fragments double-stranded and increased the amounts of available material. It was not known how many cycles of amplification would be required at this stage, since each primer pair would be expected to behave differently. It was therefore necessary directly to determine a suitable number empirically by using standard agarose gel electrophoresis to examine the reaction products after a given number

of cycles. In some cases, to avoid the accumulation of non-specific products, it was necessary to perform an initial 5 cycles of amplification with both of the primers present at 2 pmoles each. All reactions were performed using 8 μl or 12.5 % whichever was the larger but not exceeding 12 μl of the column effluent above. Reaction conditions were adjusted to 20 mM Tris pH 8.3, 50 mM NaCl, 25mM Mg^{2+}, 0.5mM dNTPs and 2.5 units of Taq DNA polymerase in a final volume of 40 μl. Apart from when an initial amplification with 2 pmoles of each primer was performed, 20 pmoles of each primer were used. Cycles of amplification were performed at 95°C for 30 seconds, 65°C for 1 minute and 72°C for 3 minutes.

For the purposes of cloning, selected cDNA was amplified as described immediately above, except that the reaction was not monitored. Instead, the number of cycles which had previously been shown to just give rise to all observable products plus another 4 cycles were performed. In addition, an extra 72°C for 10 minutes incubation was performed after the last cycle.

The products of the reaction were then prepared for directional cloning. Water was added to adjust the final reaction volume to 60 μl. Enzyme was removed by two successive phenol/chloroform extractions. The final aqueous mixture was passed through an S400 column as described above, except that it had been equilibrated with 10 mM Tris HCl pH 7.5, 50mM NaCl.

For directional cloning, advantage was taken of the different known sequences introduced at each end of the selected cDNAs by the adaptors in a modification of the method of Aslandis, C. and de Jong, P. J. (Nucl. Acids Res. 18, p6156 (1990)). Different cohesive ends were produced on each end by using the exonuclease activity of T4 DNA polymerase to resect from the 3' end, to the first T in each case. To 75 μl or 75 % of the column eluate, whichever was least, were added 9.5 μl of 100mM TrisHCl pH7.4, 100 mM MgCl2, and 9.5 μl of 0.5 mM dTTP. 16 units of T4 DNA polymerase were added and the reaction incubated in a water bath at 37°C for 30 minutes. The enzyme was removed by extracting with phenol/chloroform, twice successively. The salt of the final aqueous phase was adjusted by passing it through an S300 column (Pharmacia) equilibrated with 10 mM TrisHCl pH 7.4, 1 mM EDTA as described above.

The E.coli plasmid cloning vector pBluescript KS+ (Ālting-Meese, M. A.

and Short J. M., Nucl. Acids Res. 17 p9494) was prepared for accepting the resected cDNA by restriction cleavage at the BamHI and HindIII sites and then adaptoring the resultant cohesive ends using the specific adaptors produced by the oligonucleotide 5' AGCTCGGCTCGAGTCTG (SEQ ID: 1203) with its partially complementary oligonucleotide 5' GCGACAGACAGCAGACTCGAGCCG (SEQ ID: 1204) and the oligonucleotide 5' GATCCGGCTCGAGT (SEQ ID: 1205) with its partially complementary oligonucleotide 5' CCGAGAACACTCGAGCCG (SEQ ID: 1206). Preparation of the vector and adaptoring were performed according to standard procedures. Insertion of the cDNA was performed between the BamHI and HindIII restriction sites. Recombinant vectors were transformed into the host XL1-Blue (Bullock, W. O. et al Biotechniques 5 p376-378 (1987)) by the method of Hannahan, D. J. (Mol. Biol. 166 p577-580 (1983)). Suitable standard controls for the ligations and transformations were also included.

Post transformation procedures were as described in "Molecular Cloning", 2nd Edition (Sambrook J., Fritsch, E. F., and Maniatis, T. CSH Press (1989)). Colonies were produced by plating onto X-gal/IPTG L-agar plates containing 50μg/ml ampicillin and 10μg/ml tetracyclin. Clear colonies were picked, each into a separate well of a microtitre plate, containing 100μl of L-broth and 50μg/ml ampicillin. Growth was allowed to occur for 16 hours at 37°C. 100μl of 50% or 30% glycerol was added to plates which were archived at -20°C or -80°C, respectively.

Bacteria corresponding to those archived were used for preparing templates for sequencing by the dideoxy method (Sanger, F. Milklen, S. and Coulson, A. R. Proc. Natl. Acad. Sci. 74 p5463-5467 (1977)). Bacteria for this purpose were either grown on L-agar plates containing 50μg/ml of ampicillin, prepared at the same time as they had been grown in liquid culture, or after plating out from the archive. Alternatively, fresh liquid cultures were inoculated from the archive. In all cases, cDNA inserts were amplified for sequencing by PCR (Saiki, R. K. et al Science 239 p487-491 (1988)). PCR was either performed using bacteria directly added to the reaction, by a toothpick, or PCR was performed using 1/50th of the plasmid isolated by preparative methods (Holmes, D. S. and Quigley, M. Anal. Biochem. 114 p193 (1981)) from the bacteria in the liquid cultures or from the plates.

20 pmoles of each of the PCR primers 5' biotinylated GTAAAACGACGGCCAGT

(SEQ ID: 1207) and 5' CGAGGTCGACGGTATCG (SEQ ID: 1208) were used in 40μl reactions containing 2.5mM Mg^{2+}, 50 mM KCl, Tris-HCl pH 8.3 and 0.25 units of Amplitaq (Cetus). Reactions were performed at 95°C, for 1 minute, followed by 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 40 seconds. After the cycles, a final incubation at 72°C for 5 minutes was performed.

After PCR, standard agarose gel electrophoresis was used to determine which reactions had been successful. The biotinylated strands of successful reactions were then recovered for single-stranded sequencing by binding them to steptravidin coated beads (Dynal) and then washing, all according to the manufacturers instructions, except that the washing steps were either performed manually or performed automatically in the 96 well microtitre plate format using a Biomek robotic work-station attached to a side-arm loader (Beckman).

Dideoxy chain termination sequencing reactions were performed using the immobilised, biotinylated strands as templates and 2 pmoles of the oligonucleotide 5' CGAGGTCGACGGTATCG (SEQ ID: 1209) as primer. Reactions were performed using fluorescently-labelled terminators (Du Pont) or a fluoroscein-labelled primer (Pharmacia) according to the manufacturers instructions. Reactions were analysed using automated DNA sequencers. A Genesis 2000 was used for the "Du Pont" reactions and an A.L.F. for the "Pharmacia" reactions. Bases were assigned for the Genesis 2000 reads using the manufacturers Base Caller software. Files of called bases were then transferred to a SUN Network from an Apple Macintosh computer which had been used for base calling. Raw data from the A.L.F. reads was directly transferred to a SUN network where bases were called using the public domain "trace editor software" (TED). In both cases, files of called bases were entered into a Sybase™ database. Entering data entailed automatically removing vector and adaptor or linker sequences, but not editing ambiguous bases. After removal of the unwanted bases, files were automatically compared to other sequences in the cDNA database and the latest versions of the publically available databases, GENBANK and SWISSPROT. Searches were performed with the "basic local alignment search tool" (BLAST) (Karlin, S. and Altschul, S. F. Proc. Natl. Acad. Sci. 87 p2264-2268 (1990)).

Sequences SEQ ID Nos 1 to 610, given hereinafter, were obtained by the above procedure.

Example 2

A second method of preparing cDNA libraries for obtaining gene fragments of the invention took advantage of the PCR normalisation process described above. Standard procedures were used to prepare mRNA from RNA that had been isolated by standard caesium chloride bouyant density gradient methods from a full term human placenta. The oligonucleotide LNotdt, sequence 5' TACGTTCGACAAGCTTGAATTCGCGGCCGC$(T)_{26}$, (SEQ ID: 1210) was used at 1 μM with AMV reverse transcriptase, to prime first strand cDNA synthesis under standard conditions from 0.5 μg of the placental mRNA. Temperatures above 65°C were used to inactivate the reverse transcriptase and then the volume of the reaction made up to 100 μl with water.

PCRs were then performed in reactions containing 1 μl of the diluted cDNA, 10 mM Tris-HCl pH 8.3, 40 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM dNTPs, 10 uCi $a^{32}P$ dCTP, 1 μM each of the primers 11AD1, sequence 5' GCC(TA)(GC)CGCCGA (SEQ ID: 1211), and LNotdT and Taq DNA polymerase. An initial denaturation period of 95°c for 90 seconds was followed either by 35 cycles of standard PCR, comprising 95°C for 30 seconds, 45°C for 30 seconds and 72°C for 30 seconds or alternatively 3 cycles of the standard PCR already described followed by 27 cycles of Cot PCR during which an additional step of 72°C for 16 minutes was placed between all of the 95°C and 45°C steps of the standard PCR. The standard PCR was followed by a single 72°C for 3 minutes step while the Cot PCR was followed by one standard PCR cycle except that the 72°C incubation was performed for 3 minutes.

Products of the PCR reaction were end repaired by adding 5 units of T4 DNA polymerase to the reaction and then incubating at 37°C for 10 minutes. Enzymes were removed by phenol extraction. The cDNA was precipitated by 70% ethanol, dried and then resuspended in NotI buffer. 20 units of NotI were used to digest the cDNA under standard conditions. cDNA was again phenol extracted and ethanol precipitated. 10% of the purified NotI cut DNA were ligated to the vector pBluescript lting-Meese, M. A. and Short J. M. Nucl. Acid Res. 17 p9494 which had been prepared as standard to receive this DNA by restricting with the enzymes NotI and EcoRV. Transformation and processing of clear colonies was performed as described above except that the host E.coli strain DH5a was used in place of XL-1 Blue.

Preparation of clones for sequencing, sequencing and sequence analysis of cDNAs in clones thus-produced were performed as described in Example 1.

Sequences SEQ ID Nos 611 to 772, given hereinafter, were obtained by the above procedure.

Example 3

cDNA libraries corresponding to adult brain cortex (Clontech Laboratories, Inc., Cat No. HL10036) and adult bone marrow (Clontech Laboratories, Inc., Cat No. HL10586) prepared in lambda gt11 phage were transfected into E.coli Y1090 and plated out for colour selection of recombinant plaques ("Molecular Cloning", 2nd Edition Sambrook J., Fritsch, E. F., and Maniatis, T. CSH Press (1989)). 192 lambda Zap clones, corresponding to rhabdomyocarcoma cDNAs and a gift from C. Cooper, ICR, Sutton, were similarly plated except that the host XL-1 Blue was used.

Clear plaques from each library were resuspended in 5 μl of Tris-HCl pH 8, 1 mM EDTA. 2 μl of the resultant phage suspensions were added directly to PCRs for the purpose of amplifying the cDNA inserts for sequencing. PCR was performed as described in Example 1, except that the oligonucleotides used as primers for the lambda gt11 clones were 5' GGTGGCGACGACTCCTGGAGCCCG (SEQ ID: 1212) and 5' TTGACACCAGACCAACTGGTAATG (SEQ ID: 1213). Whichever of the oligonucleotides was to be used to prime the strand which would serve as the sequencing template was used in biotinylated form.

Preparation of clones for sequencing, sequencing and sequence analysis of cDNAs in clones thus-produced was performed as described in Example 1, except that 2 pmoles of the primers that were unbiotinylated in the PCR were used as sequencing primers.

Sequences SEQ ID Nos 773 to 1193, given hereinafter, were obtained by the above procedure.

The following are the SEQUENCE LISTINGS which comprise sequences SEQ ID Nos 1 to 1213 referred to hereinbefore. Certain of these sequences are preferred, and are listed as such after the main SEQUENCE LISTINGS.